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NEUROSURGERY

Vol. 30, No. 3, 1992
Printed in U.S.A.

C7

Experimental and clinical studies

Sustained Release of Nerve Growth Factor from Biodegradable Polymer Microspheres

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Although grafted adrenal medullary tissue to the striatum has been used both experimentally and clinically in parkinsonism, there is a definite need to augment long-term survival. Infusion of nerve growth factor (NGF) or implantation of NGF-rich tissue into the area of the graft prolongs survival and induces differentiation into neural-like cells. To provide for prolonged, site-specific delivery of this growth factor to the grafted tissue in a convenient manner, we fabricated biodegradable polymer microspheres of poly(L-lactide)co-glycolide (70:30) containing NGF. Biologically active NGF was released from the microspheres, as assayed by neurite outgrowth in a dorsal root ganglion tissue culture system. Anti-NGF could block this outgrowth. An enzyme-linked immunosorbent assay detected NGF still being released in vitro for longer than 5 weeks. In vivo immunohistochemical studies showed release over a 4.5-week period. This technique should prove useful for incorporating NGF and other growth factors into polymers and delivering proteins and other macromolecules intracerebrally over a prolonged time period. These growth factor-containing polymer microspheres can be used in work aimed at prolonging graft survival, treating experimental Alzheimer's disease, and augmenting peripheral nerve regeneration. (*Neurosurgery* 30:313-319, 1992)

Key words: Biodegradable polymer, Nerve growth factor, Parkinson's disease, Sustained release

INTRODUCTION

The grafting of dopamine-rich tissues to the basal ganglia has recently been studied in clinical and animal trials as a potential treatment for patients with Parkinson's disease (1, 3–9, 13, 16–18, 20, 22, 23, 26, 27, 31, 32, 34, 44, 46, 47). Early animal work and human studies with adrenal medulla grafts were promising (17, 34); however, later results have been equivocal in attempting to achieve the same substantial behavioral improvement in humans (1, 26). Adrenal medullary grafts survive poorly in most animal studies (13, 16, 20). Although fetal mesencephalic cells demonstrate increased survival (4, 8, 9, 18, 33, 45), the use of human fetal tissues remains problematic. Several methods aimed at enhancing the survival of the grafted adrenal medullary tissue have been proposed. One approach is the creation of a cavity in the area to be grafted, followed by the delayed implantation of the graft into this cavity (4, 8, 20, 44). The exact mechanism of the increased graft survival is unknown, but it is hypothesized to be related to the increased vascularity provoked by the lesion (44) or the release of trophic factors (4, 11). Exposing grafted adrenal medullary tissue to nerve growth factor (NGF) can also markedly enhance survival. Infusion of NGF into the area of the graft for as short a time period as 28 days could prolong chromaffin tissue survival to at least 1 year, if not longer (47). Exposure to NGF-producing C6 glioma cells enhanced both cell survival and functional recovery in the 6-OHDA rodent model of Parkinson's disease (6). Enhanced chromaffin cell survival occurs after cografting with sural nerve, known to be a rich source of NGF (27). Peripheral nerve regeneration is also markedly enhanced by NGF in animal models (40). NGF also appears useful in the treatment of experimental Alzheimer's disease (21, 24). Grafts of the male mouse submaxillary gland, a major source of NGF, can increase survival of damaged basal forebrain neurons (43).

Although NGF and other compounds have the potential to enhance graft survival, the delivery of these compounds to the target tissue poses a major challenge. Continuous infusion of substances into the brain parenchyma through a pump is possible, although pump-derived toxins can be a major problem (52). Moreover, the currently available osmotic pumps are able to release aqueous solutions for a maximum of 2 weeks. One possibility is to use sustained-release, biodegradable preparations, already shown to be efficacious for the delivery of antibiotics, anticancer agents, and contraceptives (12, 30, 36, 39, 41, 48, 50, 55). In a neurological application, a chemotherapeutic agent was incorporated into a biocompatible polymeric matrix. The drug-incorporated polymer was fabricated into solid particles and was effective in delivering site-specific chemotherapy intracerebrally in an animal tumor model (55). A biodegradable, polyanhydride polymer drug delivery system is currently under investigation in the treatment of human gliomas. Poly(DL-lactide)co-glycolide (50:50) was used as the matrix material to deliver dopamine to the dopamine-depleted striatum in a rat model of Parkinson's disease (35). Implantation of this delivery system resulted in sustained release of dopamine into the basal ganglia over a 6-week period with minimal reaction in the surrounding brain tissue. The promise of this approach led us to fabricate a slow release, biodegradable, biocompatible preparation from which NGF could be

Published by
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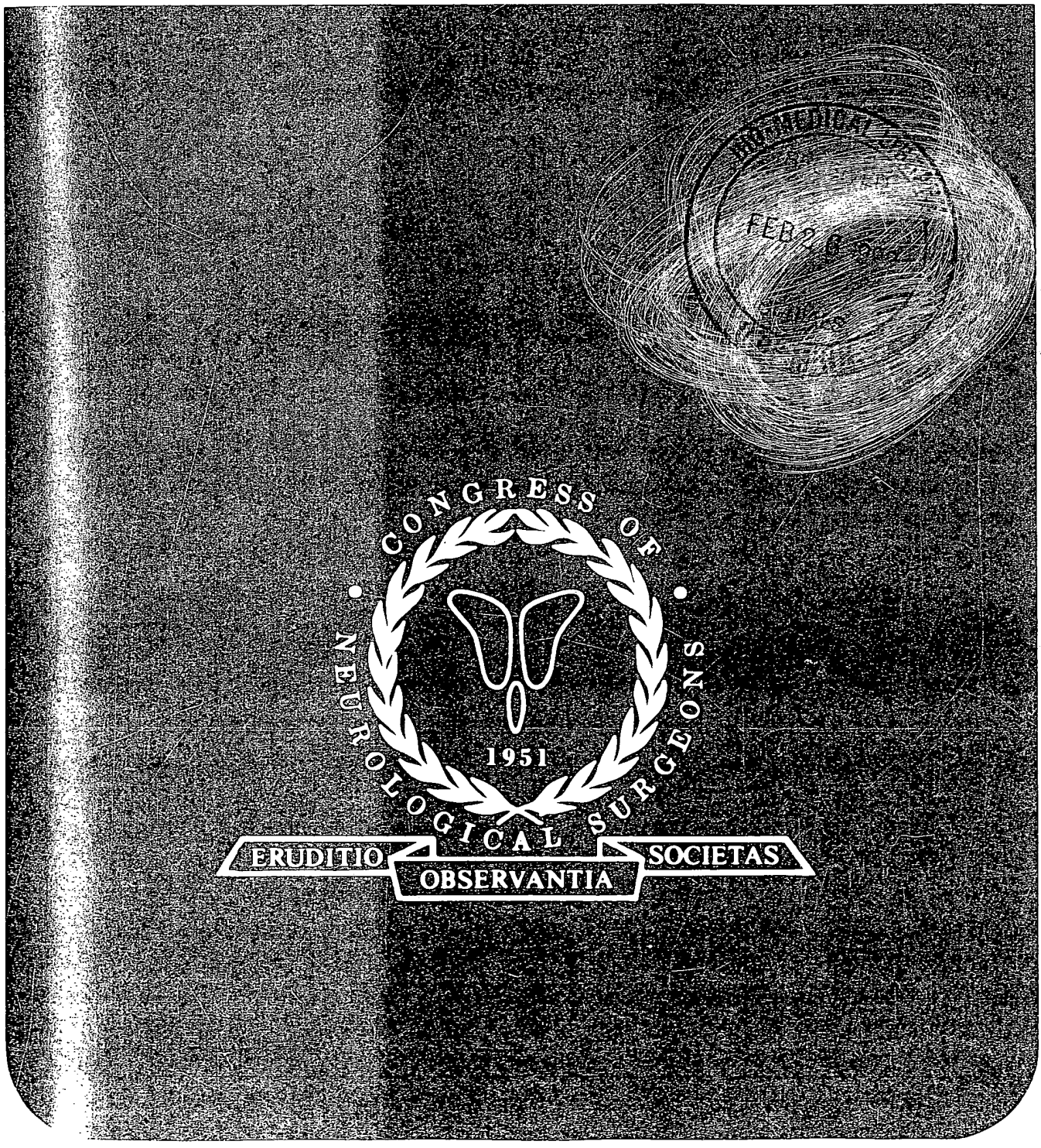
VOLUME 30/NUMBER 3

MARCH 1992

NEUROSURGERY

OFFICIAL JOURNAL OF THE CONGRESS OF NEUROLOGICAL SURGEONS



locally released into the brain over a prolonged period of time, avoiding problems inherent in grafting NGF-rich tissues or bulky pump-infusion methods.

MATERIALS AND METHODS

Microsphere preparation

Microspheres were prepared according to the method of Ogawa et al. (36). Poly(L-lactide)co-glycolide (70:30) (Polysciences, Inc., Warrington, PA) was used as the matrix material. This polymer was chosen because it is a nontoxic, biodegradable polymer from the same class of materials as bioabsorbable sutures (15). Microspheres were prepared with two NGF loadings, 0.001% and 0.013% w/w. The weighed amount of 2.5S NGF (Boehringer-Mannheim, Indianapolis, IN) was dissolved in 500 μL of 20% w/vol aqueous gelatin solution at about 40°C. The polymer (2 g) was dissolved in 5 mL of methylene chloride. The oil phase was added to the aqueous solution while it was vigorously stirred with a homogenizer at 5000 rpm, producing a water-in-oil emulsion. The emulsion was cooled to 15°C and poured into a 0.5% w/vol aqueous solution of polyvinyl alcohol with vigorous stirring. This procedure allowed the formed microspheres to harden. The organic solvent was removed by stirring the slurry in a rotary evaporator. The wet microspheres were washed three times with deionized water and dried by lyophilization.

In vitro release

The in vitro release studies were conducted in a microstirring module (Reacti-therm, Biotech, Inc., Worcester, MA). Approximately 100 mg of the NGF microspheres were dispersed in 1 mL of a buffered-balanced (pH 7.4) saline solution containing 0.5% w/vol bovine serum albumin and maintained at 37°C. At scheduled time intervals, the dispersion was centrifuged, and the release medium was removed and frozen for analysis. The microspheres were washed three times with distilled H_2O and resuspended in fresh buffer.

In vitro release of NGF was quantified by two methods— the chick dorsal root ganglion assay and the 2.5S enzyme-linked immunosorbent assay (ELISA). Originally developed by Levi-Montalcini and Hamburger (28), the bioassay detects the response of embryonic sensory ganglia to NGF. Dorsal root ganglia from 8- to 11-day-old chick embryos were carefully dissected and placed in Dulbecco's modified Eagle's medium with glutamine (Sigma Chemical, St. Louis, MO). The explants incubated overnight in a plasma clot consisting of 25 μL of thrombin 1000 U/mL diluted 10:1 with Dulbecco's modified Eagle's medium, 25 μL of the sample dissolution media (or phosphate buffer solution [PBS] with 0.5% w/vol albumin if microspheres alone were used), and 25 μL of freshly thawed chicken plasma (Cocalico Biologicals, Ephrata, PA). The cultures were then incubated at 37°C in a 5% CO_2 incubator for 20 hours. These ganglia have an absolute requirement of NGF for survival, and no neurite outgrowth was seen in the absence of NGF (Fig. 1*A*). An NGF concentration of 10 ng/mL provoked an abundant halo of neurites around the ganglion explant (Fig. 1*B*). With lower or higher NGF concentrations, the response is less, permitting quantification of the concentration of NGF. The lowest concentration of NGF detectable was 1 ng/mL.

The ELISA evaluation used an extremely sensitive sandwich enzyme immunoassay (detection limit approximately 5 pg/mL). This colorimetric assay involves the binding of an anti-NGF antibody to the well of a microtiter plate. Next, the NGF in the sample is bound to the fixed antibody. Finally, an enzyme-linked anti-NGF conjugate antibody is bound to the NGF, and the amount of the conjugate is directly proportional to the NGF content of the sample. An enzyme substrate is then added that changes color when acted upon by the enzyme, and the absorbance of the solution is measured using the substrate solution as blank. Standard microtiter 96-well plates were coated with 0.15 mL of a 0.5 mg/mL 2.5S antibody solution and incubated for 2 hours at 37°C. The plates were then washed three times with a buffer solution, and 0.1 mL of various NGF standard solutions and dilutions of the dissolution media were plated and incubated overnight at 4°C. The plates were then emptied and washed, and 0.1 mL of an antimouse-β (2.5S)–nerve growth factor-β-galactosidase (Boehringer-Mannheim) conjugate solution was pipetted into the wells and incubated for 4 hours at 37°C. The solution was removed, the plates were washed, and 0.2 mL of the enzyme substrate solution (chlorophenol red-β-D-galactopyranoside, Boehringer-Mannheim) was incubated at 37°C for 75 minutes. The absorbance was measured in a Microplate reader (Biotech, Inc.) at 540 nm. The NGF concentration in the release medium samples was determined from the standard curves constructed using NGF solutions of known concentrations.

Structure and size of microspheres

The shape, size, and structure of microspheres were studied by scanning electron microscopy (SEM). Fresh microspheres as well as microspheres suspended in buffer for 1, 2, and 4 weeks were studied. The samples were mounted onto SEM sample stubs and coated with carbon and gold (20 nm thickness) and examined under a scanning electron microscope operating at 10 keV.

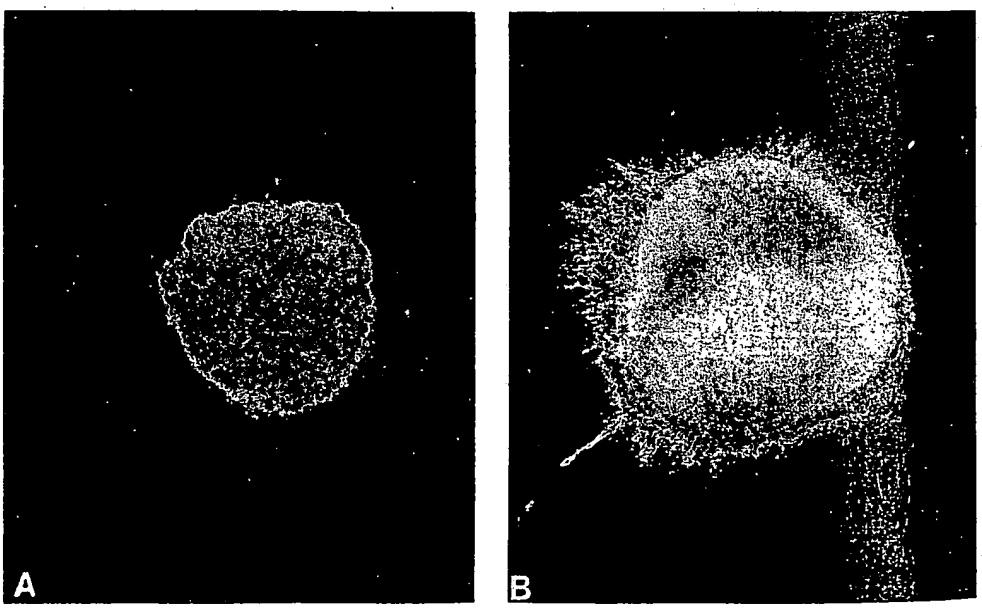


FIG. 1. Photomicrographs of 10-day-old chick dorsal root ganglia explants in tissue plasma clot after 20 hours of incubation. *A*, control with no NGF in the medium; *B*, NGF added to culture medium at 10 ng/mL.

In vivo release

Because in vitro release kinetics do not necessarily reflect in vivo release profiles, we sought to demonstrate sustained release in vivo over an extended period of time. A 3- × 1-mm plug of microspheres was implanted into the striatum of the rat (coordinates: anterior-posterior, 0.0 mm; lateral, +2.3 mm; dorsal-ventral, −5.0 mm from bregma) using a brain grafter device similar to the one described by Wyatt et al. (54). After 1 week and 4.5 weeks, the rats were killed using intracardiac perfusion with a paraformaldehyde–picric acid fixative solution. The brains were removed and placed first in the fixative for 4 hours, then transferred to a 5% w/vol sucrose buffer solution overnight. Brain sections containing the area of the implant were mounted, quick frozen in liquid nitrogen, then warmed on dry ice for 30 minutes and sectioned at 15-μm intervals. These sections were mounted on gelatin-coated slides and processed for NGF reactivity using an indirect immunofluorescence technique (10). The sections were rehydrated, covered with 10% normal goat serum in PBS, and incubated at room temperature for 30 minutes. As the primary antibody, rabbit antimouse 2.5S NGF antiserum (Collaborative Research, Bedford, MA) was diluted 1:200 in PBS containing 0.3% w/vol Triton X-100 and incubated overnight at 4°C. The sections were then washed three times (30 min each) in PBS, and incubated for 1 hour at room temperature with a fluorescein isothiocyanate-conjugated goat antirabbit secondary antibody (Boehringer Mannheim, St. Louis, MO). The sections were examined under standard epifluorescence microscopy.

RESULTS

Microsphere characteristics

SEM revealed a fairly uniform distribution of particles of the expected size with a smooth external polymer surface (Fig. 2). When suspended in the buffer solution, the microspheres exhibited a gradually increasing porosity and erosion of the polymer matrix as a function of time (Fig. 3). After 1 week, the microspheres began to lose the surface smoothness and slight indentations were observed in some particles. After suspension in buffer solution for 2 weeks, the particles were still spherical, but the surface was very porous and showed signs of degradation. By 4 weeks, there were a few remaining structures resembling microspheres. The SEM photomicrographs clearly demonstrate the gradual break-up of the microspheres. The microsphere degradation results in the release of the NGF.

Evaluation of the in vitro release of NGF

Microspheres with 0.001% w/w NGF. The release medium was removed 2, 4, 8, 24, 72, 168 (1 wk), 336 (2 wk), and 672 hours (4 wk) after contact with the microspheres, and it was subjected to the bioassay. A halo of neurite growth was produced in all cases except the 2-week and 4-week samples. Therefore, the bioassay detected NGF in the release medium for up to 1 week. One possible explanation is that all the incorporated NGF was released within a week. Alternatively, the amount of NGF released after 1 week may be below the limit of detection of the assay (approximately 1 ng/mL). In the second series of studies, the microspheres were incubated in the release medium for 24 hours, removed, washed three times with distilled H_2O, and placed directly in the tissue culture plasma clot. They provoked abundant neurite outgrowth (Fig. 4*A*). The neurites are thicker on the side of the ganglion exposed to the greatest number of microspheres. Proof that the outgrowth resulted from NGF release from the microspheres is confirmed by the observation that adding rabbit anti-2.5S NGF antibody to the culture completely blocked the effect (Fig. 4*B*).

Microspheres with 0.013% w/w NGF. The release medium was replaced at predetermined time intervals, and the NGF concentration in solution was determined by the ELISA. The results showed that the in vitro release of NGF from the polymer occurred for more than 5 weeks (Fig. 5). At each of the time points, the NGF released per day was calculated by dividing the NGF concentration in solution by the sampling interval (days). The sampling intervals were longer toward the end of the experiment, and the release medium was rarely sampled at intervals longer than 1 week. Because NGF is known to be somewhat unstable in aqueous media at 37°C (42), the amount of NGF released was likely to be higher than the amount of NGF measured.

Evaluation of the in vivo release of NGF. One week after implantation, NGF-like immunoreactivity was abundantly present in the microspheres, with aggregates of highly fluorescent microspheres present throughout the injection area (Fig. 6*A*). There was a noticeable absence of NGF-like immunoreactivity in the surrounding area, reflective of the negligible content of NGF in the normal striatum. After 4.5 weeks, the plug of fluorescent microspheres was markedly thinner,

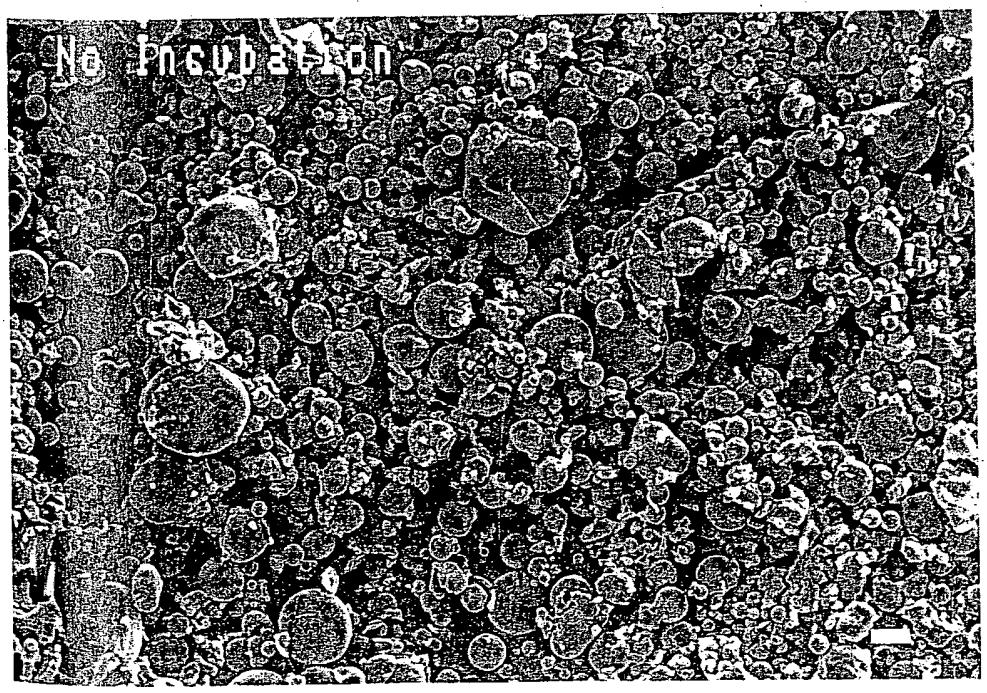


FIG. 2. Scanning electron micrograph of NGF microspheres (×500). The *bar* in the lower right corner represents 10 μm. Note the relatively constant size distribution. Most particles are less than 15 to 20 μm in diameter.

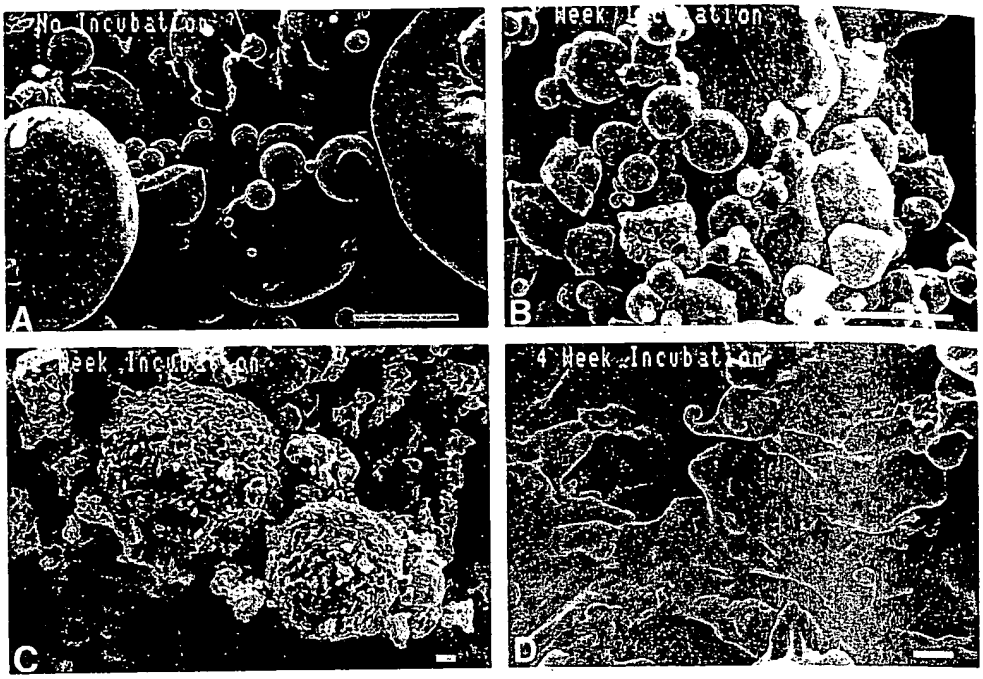


FIG. 3. Scanning electron micrographs of microsphere sample after varying intervals of incubation in the dissolution medium. *A*, no incubation (×2500). Note the smooth external surface. *B*, 1 week of incubation (×2500). The surface remains very smooth, though there are some irregularities. *C*, 2-weeks of incubation (×5000). The surface has now become very porous and irregular, and the inner matrix is eroding. *D*, 4 weeks of incubation (×1000). Few remaining structures resembling microspheres can be delineated. In *A*, *B*, and *D*, the *bar* in the lower right corner represents 10 μm, whereas in *C*, the *bar* represents 1 μm.

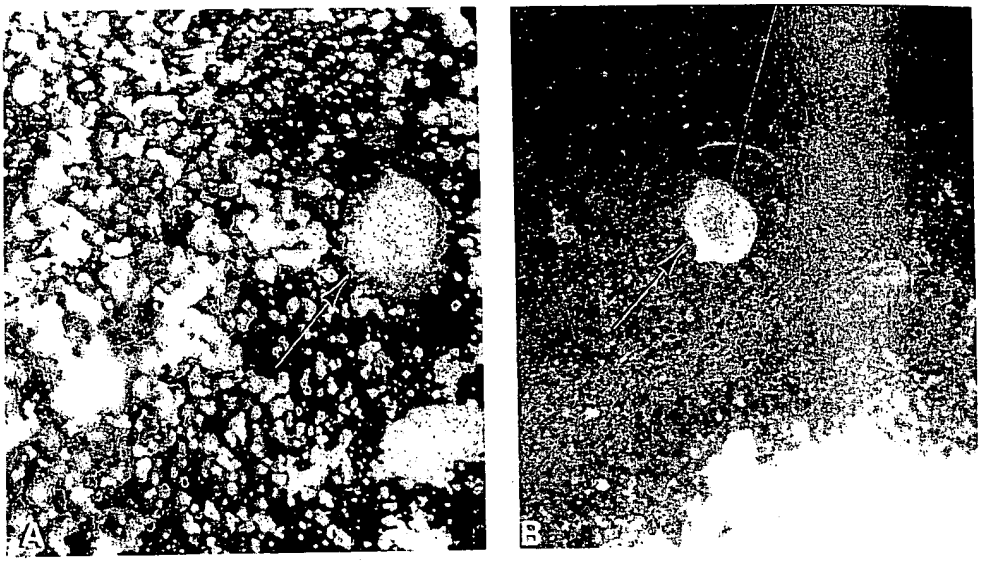


FIG. 4. Photomicrographs of dorsal root ganglia (*arrow*) exposed to microspheres for 24 hours. *A*, dorsal root ganglia with exuberant halo of neurites provoked by microspheres releasing NGF. The small particles are the added microspheres. Note how the halo of neurites is more pronounced on the side of the ganglion facing the greatest number of microspheres. *B*, dorsal root ganglia with no neurites in culture with anti-2.5S NGF antibody added to medium.

presumably because of the gradual release of NGF and degradation of the polymer (Fig. 6*B*).

DISCUSSION

A limiting problem with the use of adrenal medullary grafts has been their poor survival. Both rodents (16, 47) and primates (5, 23) have demonstrated little, if any, surviving adrenal medullary tissue after several months. Poor survival of grafted cells in patients who had received adrenal medullary autografts was also evident from autopsy reports (25, 37). Methods to promote and maintain adrenal medullary tissue are needed (3, 6, 20, 27, 47). One possibility is the use of NGF. The development and growth of adrenal medullary tissue is absolutely dependent on NGF (2, 42) and promotes neuronal-like transformation of adrenal chromaffin cells (29, 51). It has already been demonstrated that adrenal medullary cells survived in greater numbers in 6-OH dopamine-treated rats when supplied with NGF and that functional recovery improved (47).

The present study is an attempt to design a means of delivering NGF directly to the area of an intracerebral graft. Ideally, indwelling catheters or pumps of any type should be eliminated. Some biodegradable polymers provoke little, if any, tissue reaction in the brain (35, 48). Polyanhydrides, when implanted into the brain, have proven to be completely biodegradable over a period of weeks to months (48, 50). Incorporation of NGF into these polymers, however, would require heating the polymer to high temperatures, which could denature NGF. Another possibility is to use ethylene-vinyl acetate copolymer as the matrix substance. Sustained in vitro (19) and in vivo (14) release of dopamine was observed to occur from this polymer. Recently, in vitro NGF release from this polymer was demonstrated for up to 50 days in a PC12 bioassay that detects NGF levels above 1 ng/mL (38). A major limitation of this polymer is that it is not biodegradable. In our study, poly(L-lactide)co-glycolide (70:30) was chosen because it was inexpensive, of the same class of materials as bioabsorbable sutures, and provides great flexibility, in that its composition can be altered so as to vary the release rate of the incorporated compound (53). Hydrolysis of this polymer yields two normal

biological substrates—glycolic acid and lactic acid.

Microsphere structure

Because most of the microspheres were observed to be less than 10 μm in size, they would be easily injectable or implantable through a 20-gauge needle. The microspheres can also be mixed with the cells to be grafted. This process will result in site-specific delivery of a high concentration of NGF. The size of the microspheres is a function of the viscosity of the initial emulsion and the stirring rate. The external surface of the freshly prepared microspheres was very smooth (Fig. 2). Dispersion in the release medium for 2 weeks resulted in considerable loss of the surface smoothness, whereas after 4 weeks, the microspheres appeared to have completely lost their initial morphology (Fig. 3). This increasing porosity is in agreement with reports in the literature concerning using microspheres made of polymer of the same class, however, in vitro studies indicated release of NGF from the polymer more than a month later (Fig. 5). This conclusion was supported by the in vivo studies wherein NGF-like immunoreactivity was observed 4 to 5 weeks after implantation of the microspheres (Fig. 6*B*). Alterations in the polymer composition will alter the degradation rate of the polymer, however, NGF release for 1 month may be adequate to maintain adrenal medullary grafts for up to 1 year (47).

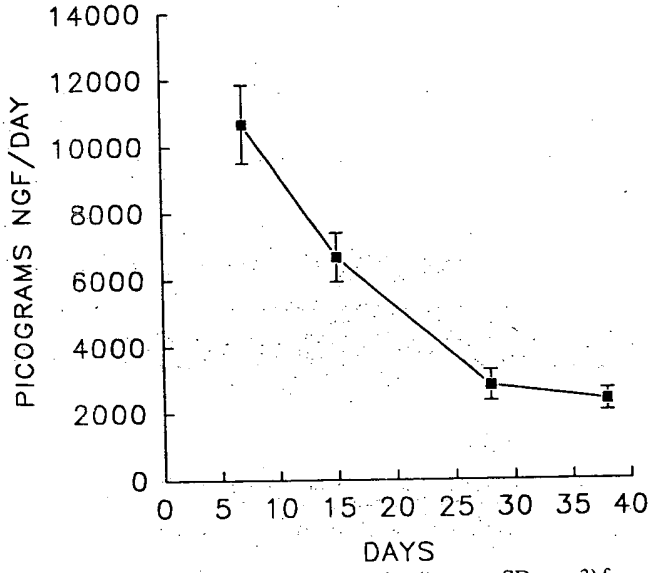


FIG. 5. Amount of NGF released per day (mean ± SD; n = 3) from 100 mg of microspheres containing 0.013% w/w NGF into 1 mL of buffer during 5-week period, as determined by NGF ELISA. Points on the graph represent the amount of NGF released per day at that point in time.

Stability of NGF in the formulation

Although the stability of NGF at different pH values is known (42, 49), little information is available about the effect of organic solvents on the protein. The process of microsphere fabrication required several steps. These steps included dissolving NGF in an aqueous solution at 40°C, addition of an organic solvent containing polymer to this aqueous solution, emulsification by vigorous homogenization, and drying by lyophilization. Some of these processes may cause denaturation of the protein, however, we were able to successfully incorporate and release NGF, as demonstrated by the bioassay and ELISA. The fraction of the added NGF that was successfully incorporated into the polymer matrix is not known. One possible approach to determine this would involve dissolving the microspheres in methylene chloride followed by extraction of NGF into water, however, this treatment method is likely to result in low recovery of NGF.

Microspheres of poly(L-lactide)co-glycolide possess the important, desirable properties in a polymer for release of biologically active molecules into the brain. These include biocompatibility, structural versatility, and biodegradation with complete absorption where the degradation products are eliminated by well-characterized metabolic pathways (12, 35, 36, 39, 50). Microspheres of this polymer have been shown to provoke little inflammatory response in the brain (35). Sustained release, easily implantable, and biodegradable delivery systems of various agents have proven extremely useful in such areas of treatment as antibiotic therapy, delivery of contraceptives, and chemotherapy. Current efforts in our laboratory are aimed at using this polymer to enhance adrenal medullary graft survival in rodent and primate models of Parkinson's disease. The development of a preparation for the intracerebral delivery of NGF would be invaluable in the continuing study of neural grafting in the treatment of Parkinson's disease and the experimental treatment of Alzheimer's disease as well as promoting peripheral and central nerve regeneration. These results demonstrate that the production of such a polymer is

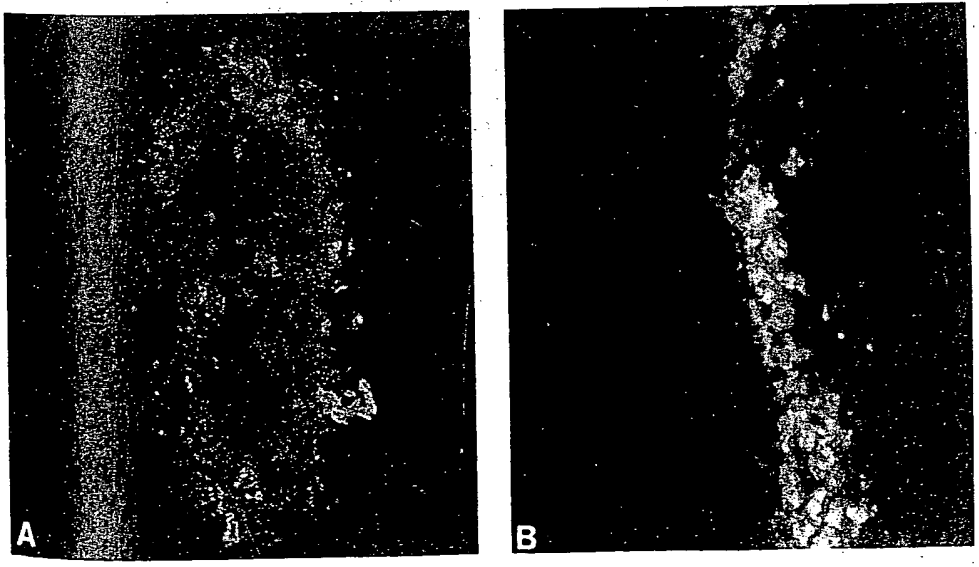


FIG. 6. Photomicrographs of rat brain slices containing microsphere implant in the basal ganglia stained with fluorescent antibody technique for NGF. *A*, 1 week after implantation there is abundant NGF-like immunoreactivity still present in the microspheres. *B*, after 4.5 weeks, the implant is much smaller in width, presumably because of reabsorption of the polymer mass, but some NGF-like reactivity is still present.

feasible and should be immediately applicable in these areas of neuroscience.

ACKNOWLEDGMENTS

This study was supported by grants from the Minnesota Medical Foundation, the Russell Sabor Foundation, the United Parkinson Foundation, and Mr. Hal Seth. Dr. Camarata is supported by a 1991 American College of Surgeons scholarship. The authors thank Mr. Mike McPhee for assistance in preparing the figures, and Dr. Patricia Tam for allowing the use of equipment for the enzyme-linked immunosorbent assay.

Presented in part at the 40th Annual Meeting of the Congress of Neurological Surgeons, Los Angeles, California, October 20–25, 1990.

Received for publication, June 10, 1991; accepted, final form, September 19, 1991.

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